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(54) Title: ASSESSMENT OF TRANS-ACTING FACTORS ALLELIC VARIATION

(57) Abstract

The present invention provides a genetic test for assaying predisposition to and/or resistance to high rates of bone turnover, development of low bone mass and responsiveness or otherwise to therapeutic modalities. This is a specific model for use in prediction of osteoporosis and likely response to preventive or therapeutic modalities. It is a general model of allelic variation in transcriptional regulators determining physiological set-points and thus susceptibility or resistance to certain pathophysiological states.

ASSESSMENT OF TRANS-ACTING FACTORS ALLELIC VARIATION

Field of the Invention

The present invention relates to a method of identifying allelic differences in trans-acting factors as a means of identifying individuals at risk to suffer from an adverse pathophysiological condition. The method of the present invention is particularly useful in assessing allelic variations in the vitamin D receptor gene and thereby predicting predisposition to low or high bone density. Moreover these variants could be used to predict long-term risk of osteoporosis as well as predicting response to different modalities of therapy. This effect is also a model of determination of predisposition to or resistance to other pathological or physiological variations due to other transcription factor gene variants and thus determining risk of disease and of response to therapy. Such transcriptional regulators could be, but not limited to, ligand-activated gene regulators such as the steroid/retinoid/thyroid hormone receptor gene family.

Background to the Invention

Vitamin D functions as a potent regulator of bone and calcium homeostasis as well as of cellular differentiation and replication in many target tissues. It acts as its dihydroxylated metabolite (1,25-dihydroxyvitamin D, or calcitriol) through the highly specific vitamin D receptor (1). This trans-acting transcriptional activator protein mediates calcitriol action in the regulation of the expression of target genes. Cloning the vitamin D receptor gene (2,3) showed it to be a member of the ligand-activated receptor superfamily that includes the receptors for steroid hormones (glucocorticoids, progesterone, estrogen, androgen, and mineralocorticoids) as well as thyroid hormones and vitamin A derivatives (4,5),

natural regulators of a large number of physiological
and developmental processes. The mechanisms by which
these receptor proteins mediate the regulation of gene
expression has been a subject of intense research.
Rare overt mutations have been identified that
compromise the function of receptors and that cause
major functional disorders in humans and animals. For
example, mutations in the vitamin D receptor gene,
resulting in vitamin D-resistant rickets (6), and in
the androgen receptor, resulting in androgen
insensitivity (7), have been reported, and in the
estrogen receptor gene an infrequent natural
polymorphism has been correlated with a high rate of
spontaneous abortion (8). However, despite a wealth of
molecular information, little is known of the potential
contribution of natural allelic variation in receptor
genes to diversity of response to steroidal hormones in
normal physiology and in disease states.

    Osteoporosis is a major public health problem
among the elderly in most Western countries involving
both enormous health care costs and debilitating
long-term effects (Riggs NEJM). Since therapy of
established osteoporosis remains far from satisfactory,
prevention is the best choice. Preventative strategies
for osteoporosis must focus upon development of peak
bone density in early adulthood and minimisation of
age-related and postmenopausal bone loss. Evidence
from twin and family studies have shown strong genetic
effects on peak bone density that is modifiable by
hormonal factors, nutrition and life style (Kelly et
al, OI). Twin studies have demonstrated that
monozygotic twin pairs have a much greater concordance
for axial and appendicular bone density than do
dizygotic pairs. Analysis of these data indicated that
these genetic factors account for approximately 75% of
the total variation on bone density. This effect has

been confirmed in mother-daughter pair studies. The present inventors analysed the potential mechanisms of this genetic effect in the twin model. The present inventors found that the genetic effect was apparent in certain biochemical indices of bone turnover, such as osteocalcin, a marker of bone formation. Moreover amongst dizygotic twins the higher osteocalcin level was associated with the lower bone density. The present inventors have also found that the genetic effect can be shown with equal strength in another marker of bone formation, i.e., procollagen type I C-terminal propeptide and less strongly in a marker of bone breakdown, collagen type I C-terminal telopeptide. Under normal circumstances bone formation and bone breakdown are tightly linked or "coupled" in the twin physiological process of bone turnover. Thus the somewhat surprising results from the twin studies indicate that the bone formation markers, as markers of bone turnover, predict bone density and that genetic regulation of bone turnover is the pathway of the strong genetic effect on bone density.

The cross-sectional data on bone density in twins suggested that a single gene or set of genes is responsible for the genetic effect on bone density. However, it was unknown how this effect is mediated and which gene or genes influence bone density. In recent studies, using restriction fragment length polymorphism, the present inventors have shown common allelic variation in the vitamin D receptor (VDR) locus predict osteocalcin, independent of age, sex or menopausal status (Morrison et al, PNAS). The vitamin D receptor gene, as the active hormonal form of vitamin D (1,25-dihydroxyvitamin D), is an important central regulator of bone and calcium homeostasis modulating intestinal calcium absorption, bone formation, recruitment of the bone resorbing cell (osteoclast) and

bone resorption per se as well as parathyroid hormone production and vitamin D's own activation in the kidney. Because of the likelihood that any alterations in the receptor for the active hormonal form of vitamin

5   D could have such wide effects, the effect of these common VDR gene alleles on bone density was examined using a twin model. In the twin model, within-pair comparisons eliminate age and various cohort effects as confounders.

10   The studies have shown that commc allelic variants in the VDR gene predict differences in bone density and account for 50-75% of the total genetic determination of bone density in the spine and hip.

It is believed that this a clear example that

15   genotypic variations in transcriptional regulators of genes encoding regulatory and/or structural proteins, determine physiological set-points and predisposition to pathophysiological states with implications for susceptibility to disease and for determining likely

20   responses to therapy.

Accordingly in a first aspect the present invention consists in a method of assessing in an individual's predisposition to a pathophysiological state and/or likely response to therapy comprising

25   analysing genotypic variations in transcriptional regulators of genes encoding regulatory and/or structural proteins.

In a second aspect the present invention consists in a method of predicting predisposition of an

30   individual to low or high bone density comprising analysing allelic variation within the vitamin D receptor gene of the individual.

In a preferred embodiment of the present invention the analysis comprises restriction fragment length

35   polymorphism using endonuclease digestion.

In a further preferred embodiment of the present invention a segment of the vitamin D receptor is amplified using polymerase chain reaction prior to endonuclease digestion.

In yet a further preferred embodiment of the present invention the endonuclease is selected from the group consisting of Bsml, Apal, EcoRv and Taql, and is most preferably Bsml.

In another preferred embodiment of the present invention the segment of the vitamin D receptor is amplified using a pair of primers selected from the group consisting of

5' - CAACCAAGACTACAAGTACCGCGTCAGTGA - 3'

and 5' - AACCAGCGGAAGAGGTCAAGGG - 3';

and 5' - CAGAGCATGGACAGGGAGCAAG - 3'

and 5' - GCAACTCCTCATGGCTGAGGTCTCA - 3'.

In a second aspect the present invention consists in a primer pair derived from the sequence of the VDR gene shown in Table 5 for use in amplifying a segment of the VDR gene using polymerase chain reaction, the segment including at least one of the Bsml, Apal or Taql cut sites as shown in Table 5.

In a preferred embodiment of this aspect of the present invention the primer pair is

5' - CAACCAAGACTACAAGTACCGCGTCAGTGA - 3' and

5' - AACCAGCGGAAGAGGTCAAGGG - 3', or

5' - CAGAGCATGGACAGGGAGCAAG - 3' and

5' - GCAACTCCTCATGGCTGAGGTCTCA - 3'.

The allelic makeup of other transacting factors which may be assessed include oestrogen and androgen receptors to determine risk of osteoporosis and/or ischaemic heart disease. The allelic makeup of the androgen receptor may be also used to assess risk and responsiveness to therapeutic intervention in skin diseases. The allelic makeup of the glucocorticoid receptor and the retinoic acid receptor can be

determined to assess risk of osteoporosis. The allelic makeup of the mineralocorticoid receptor can be determined to assess risk of hypertension and the allelic makeup of proto-oncogenes can be determined to assess cancer risk. Tissue specific regulators can also be assessed to determine osteoporosis/cancer risk.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples and figures in which:-

Figure 1 shows lumbar BMD differences in twin pairs according to vitamin D receptor alleles.

Figure 2 shows a map of the vitamin D receptor gene from exon 7 to the start of the 3' non-coding sequence of exon 9 showing the location of polymorphic restriction enzyme sites used in this study and the fragments amplified by PCR used to detect the RFLPs. Asterisk denotes polymorphic site while absence of the asterisk indicates an invariant site.

Figure 3 shows bone mineral density is different in VDR genotypes: female subjects. Data shows the population mean ± standard error mean. p values are for the pairwise two-sided Students t-tests for the groups.

Figure 4 shows the genetic effect on bone mass at the lumbar spine is also apparent in males. Symbols are as for Figure 3.

Figure 5 shows age related regression of lumbar spine bone mineral density and intersection with the fracture threshold according to genotype.

Figure 6 shows age related regression of femoral neck bone mineral density and intersection with the fracture threshold according to genotype.

Figure 7 shows bone density differences between twin pairs with respect to zygosity and concordance for VDR alleles. Bone density in the lumbar spine and

proximal femur is expressed at the within pair percent
difference in bone density in MZ and DZ twin pairs and
according to whether the DZ twin pairs are concordant
or discordant for the VDR. The DZ twins concordant for
5   the VDR alleles are not significantly different from
the MZ twins at any site, while the discordant DZ twins
are significantly different (ANOVA) from both of these
groups at each site. The difference between the total
DZ group and those concordant for the VDR alleles
10   compared with the MZ twins indicates that 75%; 48%, 59%
and 90% of the genetic effect can be explained by the
VDR alleles at the lumbar spine, femoral neck, Ward's
triangle and trochanteric region of the proximal femur
respectively. Genotype for another developmental
15   transcriptional activator, retinoic acid receptor-α
(21q7), did not predict ΔBMD at any site.
       Figure 8 shows the difference in bone density
between dizygotic twin pairs with respect to degree of
discordance for VDR. The difference in bone density
20   between twin pairs is plotted in three groups; 0 -
complete concordance, 1 - one allele different, 2 -
both alleles different. Panels A, B, C and D show the
analyses for the VDR gene in the lumbar spine femoral
neck. Ward's triangle and trochanteric region
25   respectively. Regression analysis of this effect shows
significant relationships at the lumbar spine
(p=0.0001), Ward's triangle (p=0.006) and trochanteric
region (p=0.034) and borderline at the femoral neck
(p=0.055). Using the sib-pair variance approach,
30   significant relationships were observed between the
squared difference in bone density within each twin
pair (Δ^2) and concordance for the VDR gene alleles at
the lumbar spine, femoral neck and Ward's triangle and
borderline at the trochanteric region of the proximal
35   femur.

Lumbar spine Δ^2 = 0.015+0.038 * Degree of discordance (r=0.43, p=0.001)

Femoral neck Δ^2 = 0.015 + 0.016 * Degree of discordance (r=0.29, p=0.034)

Ward's triangle Δ^2 = 0.017 + 0.026 * Degree of discordance (r=0.34, p=0.01)

Trochanteric region Δ^2 = 0.015 + 0.015 * Degree of discordance (r=0.27, p=0.05)

Figure 9 shows higher bone mineral density associated with the b allele of the VDR gene.

A. Lumbar spine bone mineral densities of dizygotic twin pairs discordant for Bsm-1 alleles (n=22) are plotted as twin and co-twin according to genotype. Lines connect bone mineral density values for a twin pair. In 21 of 22 pairs, the twin carrying extra presence of the site (b) alleles has the higher bone mass (open circles). A single twin pair (black circles) has the reverse situation.

B. Bone mineral density at the lumbar spine amongst unrelated premenopausal females according to VDR genotype. One of each (premenopausal female) MZ and DZ twin pair was randomly selected for this analysis and the numbers of individuals are shown for each group. It is clear that the BB genotype has a lower mean BMD at the lumbar spine while the bb group has the higher mean BMD. The magnitude of this effect can be appreciated in relation to the standard deviation of bone density in an age-matched population of about 0.11gm/cm^2 at each site. The mean $\pm$SE is plotted and significance of the difference between groups was calculated by ANOVA. The pair-wise comparisons were made by unpaired Student's-tests. the different groups were not significantly different for age, height or weight.

Figure 10 shows the results of calcitriol therapy in individuals of different genotype.

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STUDY 1

Methods

Two hundred eighty-eight subjects recruited for
epidemiological studies of bone density were included
in the study. All subjects were recruited from the
Sydney metropolitan area, latitude 33°52'S, a region of
high sunlight incidence. Ninety-one subjects of
Caucasian British-Australian origin (United Kingdom and
Irish background) with restriction fragment length
polymorphism (RFLP) data for the three endonucleases
had serum osteocalcin data available. None of the
subjects was taking medication known to cause bone
disease or influence osteocalcin levels. All subjects
were Caucasian and had normal renal function as
determined by serum creatinine.

Serum was collected in the morning after overnight
fast, and none of the subjects was treated with
calcitriol prior to venipuncture. Serum osteocalcin
was determined by an in-house radioimmunoassay based on
rabbit anti ovine osteocalcin (11). The normal range
of osteocalcin found with this assay is 3-18ng/ml when
purified ovine osteocalcin is used. Osteocalcin
determinations were made prior to, and independently
of, the RFLP analysis and the results were stored in a
coded fashion.

DNA Analysis. The probe used to identify RFLPs
was a 2.1-kilobase-pair fragment of the vitamin D
receptor cDNA (3,18) covering the entire coding region
but lacking the 3' untranslated portion of the mRNA.
Extraction of DNA from blood and Southern blotting were
done by standard methods. Restriction enzymes were
obtained from Pharmacia-LKB and New England Biolabs and
used according to the suppliers' specifications.

Statistical Methods. The relative association of
the RFLP markers was assessed statistically for
deviation from the null hypothesis of free association

by using contingency tables and x^2 tests. The Statview-plus-graphics statistical package (Abacus Concepts, Berkeley, CA) run on a Macintosh SE/30 computer was used for analysis of variance (ANOVA).

5 Fisher's protected least-significant-difference (PLSD) test was used to assess the relationship between RFLP and serum osteocalcin. Significance levels quoted are for the initial F tests on the null hypothesis (no difference between the means) of the overall effect and

10 for the confidence level of the pairwise comparison of the continuous variable means of each categorical (RFLP) class.

Each RFLP marker system was considered separately for its association with osteocalcin serum

15 concentrations by ANOVA comparing categorical classes (RLFPs) against the continuous variable (osteocalcin). The osteocalcin values (ng/ml) were not normally distributed, and so nonparametric analysis was performed as well as logarithmic transformation as ln(1

20 + osteocalcin).

<u>Results</u>

Two previously unreported frequent RFLPs (detected by Bsm I and EcoRV) were found by using the vitamin D receptor cDNA probe, in addition to a previously

25 reported RFLP detected by Apa I (18). The RFLPs were coded as Aa (Apa I), Bb (Bsm I) and Ee (EcoRV), where the uppercase letter signifies absence of the site and lowercase signifies presence of the site. The Mendelian nature of the RFLPs was verified by family

30 studies (data not shown). The frequencies of these RFLPs in 266 unselected volunteers unrelated to this study are shown in Table 1. The genotypes of 182 individuals were assessed with all three RFLPs (Table 2). They demonstrated a strong degree of

35 coassociation, indicating linkage disequilibrium at this locus. The RFLPs were highly associated such that

AA was found with BB and EE at frequencies of 83% and 92%, respectively; correspondingly, aa was found with bb and ee at frequencies of 61% and 72%, respectively. The subsequent functional analysis does not depend on haplotyping; however, only two of a possible eight haplotypes are needed to account for 53.2% of the test population. The apparent homozygotes define the most frequent possible haplotypes as a b e and A B E (Table 2).

TABLE 1

Frequencies of RFLP Alleles

Enzyme	Allele 1	Allele 2	n*
Apa I	A, 0.494	a, 0.506	256
Bsm I	B, 0.439	b, 0.560	182
EcoRV	E, 0.490	e, 0.510	255

* No. of individuals tested.

TABLE 2

Frequencies of RFLP Genotypes

Homozygotes	n*	Heterozygotes	n*
aa bb ee	26	Aa Bb Ee	72
AA BB EE	19	AA Bb EE	13
AA bb EE	2	aa Bb ee	8
aa BB ee	2	Aa Bb ee	8
		Aa bb Ee	7
		Aa bb ee	4

* No. of individuals per 182 tested with all three RFLPs (heterozygote classes with <4 individuals have been excluded).

TABLE 3

Osteocalcin Values and Vitamin D Receptor Alleles

RFLP	n	Median	Mean	SD	SE	Sig.1		Sig. 2		P value
Bsm 1										
BB	16	16.8	2.86	0.45	0.11	BBvsbb	0.00005	BBvsBb	0.0001	0.0001
Bb	46	8.9	2.12	0.58	0.09					
bb	25	8.8	1.97	0.71	0.14					
Total n	87									
Apa 1										
AA	25	14.0	2.53	0.63	0.13	AAvsaa	0.001	AAvsAa	0.04	0.0023
Aa	45	9.3	2.18	0.59	0.09					
aa	20	7.5	1.83	0.78	0.18					
Total n	90									
EcoRV										
EE	26	14.0	2.53	0.63	0.12	EEvsee	0.015	EEvsEe	0.015	0.0153
Ee	45	8.8	2.11	0.60	0.09					
ee	18	10.5	2.02	0.83	0.20					
Total n	89									

Osteocalcin values among 91 Caucasian subjects of British-Australian origin (United Kingdom and Irish background) were analyzed with respect to the three informative RFLPs. As the osteocalcin values were not normally distributed, they were logarithmically transformed prior to statistical analysis. Median, median of serum osteocalcin values; Mean, mean of log-transformed values [ln(osteocalcin + 1)]; n, number of subjects; SD, standard deviation; SE, standard error of the mean; Sig., significance (probability that such a difference could occur by chance) referring to the difference between the means of the homozygotes (Sig. 1) and to the difference between the homozygote (absence of RFLP site) and the heterozygote (Sig. 2). P value is for the F test on the overall effect.

SUBSTITUTE SHEET

TABLE 4

Distribution of Subjects with respect to

Age, Sex, and Menopausal Status with respect to RFLPs

	Bsm 1			Apa 1			EcoRV		
	BB	Bb	bb	AA	Aa	aa	EE	Ee	ee
No. of subjects	16	46	25	25	45	20	26	45	18
Age, years	52±14	50±14	44±14	51±15	50±15	45±12	49±14	50±15	46±12
No. female	16	38	20	22	36	18	23	38	15
Postmenopausal	10	18	7	13	17	6	10	22	7
Premenopausal	6	20	13	9	19	12	13	16	8
No. male	0	8	5	3	9	2	3	7	3

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The relationship between RFLPs and serum
osteocalcin was analyzed in the 91 normal subjects
with serum osteocalcin data (Table 3).   The
distribution of this population with respect to age,
5   sex, and menopausal status is shown in Table 4.   Age
was not significantly related to any RFLP genotype.
The osteocalcin levels of the Bsm I BB group are
significantly higher than those of the Bsm I bb group
(P=0.0001).   The other RFLPs show the same effect with
10   highly significant P values for the Apa I allele
system (AA versus aa, P<0.0025) and a weaker P value
for the EcoRV RFLP (EE versus ee, P=0.015).   With all
three RFLPs the absence of restriction-site alleles
(A, B, E) is associated with high osteocalcin levels
15   and the presence of restriction-site alleles (b, a,
and e, respectively) with low osteocalcin levels: BB,
16.8ng/ml;   Bb, 8.9ng/ml;   and bb, 8.8ng/ml (medians).
Nonparametric statistical analysis (Kruskal-Wallis) of
raw osteocalcin values gave essentially the same
20   results as ANOVA: Apa I, P - 0.0016;   Bsm I,
P=0.0001;   EcoRV, P=0.0044.

Since the Bsm I and Apa I RFLPs were the most
predictive, the population was subdivided according to
the nine possible combinations of these alleles.   This
25   produced a clear separation of the serum osteocalcin
values according to genotype (Fig. 1).   Since the
weaker association of the EcoRV marker may be
determined by its disequilibrium with the other
markers, we examined the distribution of Apa I and Bsm
30   I alleles and osteocalcin values within individuals
with the EE genotype (Fig. 2).   The Bsm I marker
essentially dictated the inferred haplotypes and their
associated osteocalcin values (P=0.003).

The genotype prediction of serum osteocalcin
35   levels was maintained for Bsm I and Apa I when males
(n=14) were excluded (Bsm I, P=0.0001;   Apa I,

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P=0.0034; ANOVA values for the overall effect).
Menopause has bee⸱ associated with an increase in
osteocalcin valu⸱⸱⸱ with a wide variation in
osteocalcin valu⸱⸱ being observed in the early

5   postmenopausal years (19-21). Therefore the role of
menopausal status was assessed by multiple regression
analysis and analysis of covariance including age,
menopausal status, and Bsm I genotype. Menopausal
status was a weaker determinant of serum osteocalcin

10   concentrations than Bsm I polymorphism (r=-0.44,
P<0.001). Two-factor ANOVA yielded the same result;
Bsm I, P=0.0002; m⸱⸱⸱opausal status, P=0.24.
Analyzing premenopa⸱   and postmenopausal women
separately did not a⸱   ⸱ the results, and genotype was

15   a stronger predictor   ⸱ menopausal status (Fig. 1).

STUDY 2

Materials and Methods

Subjects

    Subjects were 535 un⸱⸱⸱lated volunteers (447

20   females and 88 males) who ⸱ ⸱ enrolled in studies of
the effect of genetics on b⸱⸱ne density. The subjects
were obtained from requests through the media in the
Sydney metropolitan area. The mean ages of the
subjects were 51.4±13.8yr (mean±SD; range 20-84yr) for

25   females and 40.6±16.0yr (20-79yr) for males. Subjects
in this analysis were of Caucasian British-Australian
origin (United Kingdom and Irish background).
Menopausal status was confirmed by the presence of
elevated FSH and LH and low estradiol levels, with an

30   absence of menses for at least 12 months. Subjects
with a history of bone disease, illness, bilateral
ovarectomy or drug use (including hormone replacement
therapy) which could affect bone turnover and bone
density were excluded from this study.

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<u>Bone Mineral Density Analysis</u>

Bone mineral density (BMD), expressed as an area
density in g/cm^2, was measured in the lumbar spine
(L2-4) and femoral neck using either dual photon
absorptiometry or dual energy x-ray absorptiometry
(Lunar DP3 or DEXA, respectively, Lunar Radiation NCo.
Madison, WI) as previously described (Pocock et al.
1987).

<u>DNA Analysis: PCR (Polymerase chain Reaction) and
RFLP Analysis using Endonuclease Digestion</u>

Blood was collected into heparin treated tubes
and leukocytes separated by sedimentation trough
physiological saline solution in a clinical
centrifuge. Purified leukocytes were lysed in
leukocyte lysis buffer (10mM Tris-HCl, pH7.4,
physiological saline and 0.5% w/v sodium dodecyl
sulphate). Lysate was treated with proteinase K
(Applied Biosciences, Palo Alto USA) at 50 ug/ml for 2
hour at 65 Celsius. DNA was extracted by repetitive
phenol chloroform solvent extraction as described in
Maniatis et al. and ethanol precipitated prior. DNA
was redissolved in TE buffer (10mM Tris-HCl, 1mM EDTA,
pH 8.0) and quantitated by ultraviolet absorbance at
260 Nm.

The vitamin D receptor gene from exon 7 to the
3'-untranslated region was sequenced. The sequence is
set out in Table 5.

Four oligonucleotide primers were synthesized to
amplify the 3' flanking region of the VDR gene.
Detection of the BsmI site was facilitated by
amplifying a region spanning the site, with one primer
originating in exon
7(5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3') and the other
in intron 8(5'-AACCAGCGGAAGAGGTCAAGGG-3') producing a
825 base pair fragment. Detection of ApaI and TaqI
sites was facilitated using a single amplification one

18   TABLE 5   _   1/5

Sequence Range: 1 to 2169

```
          10           20           30           40           50
     *     *     *     *     *     *     *     *     *     *
   CAACC AAGAC TACAA GTACC GCGTC AGTGA CGTGA CCAAA GGTAT GCCTA GACTC
   GTTGG TTCTG ATGTT CATGG CGCAG TCACT GCACT GGTTT CCATA CGGAT CTGAG
                        primer 490  Exon 7    |  Intron 7


          60           70           80           90          100          110
     *     *     *     *     *     *     *     *     *     *     *
   CACCT CCTGG GGAGT CTTTT TCAGC TCCCA GATTC TGGCT CCACC CGTCC TGGGG
   GTGGA GGACC CCTCA GAAAA AGTCG AGGGT CTAAG ACCGA GGTGG GCAGG ACCCC


         120          130          140          150          160
     *     *     *     *     *     *     *     *     *     *
   TTTGG CTCCA ATCAG ATACA TGGGA GGGAG TTAGG CACCA ACAGG GAGAG AAGGG
   AAACC GAGGT TAGTC TATGT ACCCT CCCTC AATCC GTGGT TGTCC CTCTC TTCCC


         170          180          190          200          210          220
     *     *     *     *     *     *     *     *     *     *     *
   CGAGG GTCAG ACCCA TGGGG TTGGA GGTGG GTGGG CGGCT CCTCA GCTCT TGCCC
   GCTCC CAGTC TGGGT ACCCC AACCT CCACC CACCC GCCGA GGAGT CGAGA ACGGG


         230          240          250          260          270
     *     *     *     *     *     *     *     *     *     *     *
   GCAGT ACCTG GCCAT TGTCT CTCAC AGGCC GGACA CAGCC TGGAG CTGAT TGAGC
   CGTCA TGGAC CGGTA ACAGA GAGTG TCCGG CCTGT GTCGG ACCTC GACTA ACTCG
                        Intron 7    |  Exon 8      primer 757


         280          290          300          310          320          330
     *     *     *     *     *     *     *     *     *     *     *
   CCCTC ATCAA GTTCC AGGTG GGACT GAAGA AGCTG AACTT GCATG AGGAG GAGCA
   GGGAG TAGTT CAAGG TCCAC CCTGA CTTCT TCGAC TTGAA CGTAC TCCTC CTCGT
                     primer 758               primer 456


         340          350          360          370          380
     *     *     *     *     *     *     *     *     *     *
   TGTCC TGCTC ATGGC CATCT GCATC GTCTC CCCAG GTATG GGGCC AGGCA GGGAG
   ACAGG ACGAG TACCG GTAGA CGTAG CAGAG GGGTC CATAC CCCGG TCCGT CCCTC
                                 Exon 8   |  Intron 8


     >Sac1
        |
         390          400          410          420          430          440
     *     *     *     *     *     *     *     *     *     *
   GAGCT CAGGG ACCTG GGGAG CGGGG AGTAT GAAGG ACAAA GACCT GCTGA GGGCC
   CTCGA GTCCC TGGAC CCCTC GCCCC TCATA CTTCC TGTTT CTGGA CGACT CCCGG


         450          460          470          480          490
     *     *     *     *     *     *     *     *     *
   AGCTG GGCAA CCTGA AGGGA GACGT AGCAA AAGGA GACAC AGATA AGGAA ATACC
   TCGAC CCGTT GGACT TCCCT CTGCA TCGTT TTCCT CTGTG TCTAT TCCTT TATGG


         500          510          520          530          540          550
     *     *     *     *     *     *     *     *     *     *
   TACTT TGCTG GTTTG CAGAG CCCCT GTGGT GTGTG GACGC TGAGG TGCCC CTCAC
   ATGAA ACGAC CAAAC GTCTC GGGGA CACCA CACAC CTGCG ACTCC ACGGG GAGTG
```

                                           19   2/5

              560   570   580   590   600
         *   *   *   *   *   *   *   *
    TGCCC TTAGC TCTGC CTTGC AGAGT GTGCA GGCGA TTCGG TAGGG GGGAT TCTGA
    ACGGG AATCG AGACG GAACG TCTCA CACGT CCGCT AAGCC ATCCC CCCTA AGACT
              primer 834

                                              Polymorphic site   >Bsm1
                                                                   |
      610   620   630   640   650   660
     *   *   *   *   *   * |*   *   *
    GGAAC TAGAT AAGCA GGGTT CCTGG GGCCA CAGAC AGGCC TGCGC ATTCC CAATA
    CCTTG ATCTA TTCGT CCCAA GGACC CCGGT GTCTG TCCGG ACGCG TAAGG GTTAT

                  670   680   690   700   710
             *   *   *   *   *   *   *   *
    CTCAG GCTCT GCTCT TGCGT GAACT GGGCT CAACA TTCCT GTTAT TTGAG GTTTC
    GAGTC CGAGA CGAGA ACGCA CTTGA CCCGA GTTGT AAGGA CAATA AACTC CAAAG

      720   730   740   750   760   770
     *   *   *   *   *   *   *   *
    TTGCG GGCAG GGTAC AAAAC TTTGG AGCCT GAGAG ATGGT TCTGC CTATA TAGTT
    AACGC CCGTC CCATG TTTTG AAACC TCGGA CTCTC TACCA AGACG GATAT ATCAA

                  780   790   800   810   820
             *   *   *   *   *   *   *   *
    TACCT GATTG ATTTT GGAGG CAATG TGCAG TGACC CTTGA CCTCT TCCGC TGGTT
    ATGGA CTAAC TAAAA CCTCC GTTAC ACGTC ACTGG GAACT GGAGA AGGCG ACCAA

      830   840   850   860   870   880
     *   *   *   *   *   *- *   *   *
    AGAGG TGAGA AGAGG GAGAA AAGGC CGAAG AGAAG TTATT GTGAC CTTGG GACAT
    TCTCC ACTCT TCTCC CTCTT TTCCG GCTTC TCTTC AATAA CACTG GAACC CTGTA

                  890   900   910   920   930
             *   *   *   *   *   *   *   *
    GATGT CGGTG ATGAG GTCCA AAGAG GGGCG GCCCT GCCTC AGCCT GTGCT AGTGG
    CTACA GCCAC TACTC CAGGT TTCTC CCCGC CGGGA CGGAG TCGGA CACGA TCACC

      940   950   960   970   980   990
     *   *   *   *   *   *   *   *
    CCTGT GCCCA GGGAT GCTTT CCTGG ACTGG AGGCT CAAGG AATGG AGATG GCTCC
    GGACA CGGGT CCCTA CGAAA GGACC TGACC TCCGA GTTCC TTACC TCTAC CGAGG

                  1000   1010   1020   1030   1040
             *   *   *   *   *   *   *   *
    TCTAC CCCTG CCCAG CCAGC CTTCT CTCAT TCATT CATCC ACTTA GCAAC AATTT
    AGATG GGGAC GGGTC GGTCG GAAGA GAGTA AGTAA GTAGG TGAAT CGTTG TTAAA

                             >Kpn1
      1050   1060 | 1070   1080   1090   1100
     *   *   * | *   *   *   *   *   *
    ATTGA GCACC TATTA GGTAC CAGGC ACTAT GCTAG GTACT GGGGT TCAGC AGCAA
    TAACT CGTGG ATAAT CCATG GTCCG TGATA CGATC CATGA CCCCA AGTCG TCGTT

20

```
                                    >Hind3
                                      |
        1110          1120          |1130        1140         1150
    *           *            *     | *    *          *           *           *
  ATGGG ACACA GGCTC CTCTC CCATG AAGCT TAGGA GGAAA CATTA AACAA ATGTT
  TACCC TGTGT CCGAG GAGAG GGTAC TTCGA ATCCT CCTTT GTAAT TTGTT TACAA


             >Ase1                           >Dra1
               |                               |
   1160        |   1170        1180         1190        1200         1210
    *          |  *          *           *    *           *            *
  ATTTA ATTAT TAATT CCTAA CAAGG CAAGA GTTTT AAAAA TAAAG TAAGT GATGC
  TAAAT TAATA ATTAA GGATT GTTCC GTTCT CAAAA TTTTT ATTTC ATTCA CTACG


          1220          1230          1240         1250         1260
    *           *            *           *    *           *            *
  TACAG AAGGG TAGAA TAGAA GGAGG GAAGC TGACG TGGTC TGGGC TACAG AGGTA
  ATGTC TTCCC ATCTT ATCTT CCTCC CTTCG ACTGC ACCAG ACCCG ATGTC TCCAT


                                                              >Sau3A1
                                                                 |
        1270          1280          1290         1300         1310        1320
    *           *            *           *    *           *            *
  GAGTG TTGCC AGGAA TGGCC TTTTG GAGGA AGACC TTTTA AGCTG TTATC CAAAG
  CTCAC AACGG TCCTT ACCGG AAAAC CTCCT TCTGG AAAAT TCGAC AATAG GTTTC


             1330          1340          1350         1360         1370
    *           *            *           *    *           *            *
  GATCA GTAAG AGTCT GGCAA AGATA GCAGA GCAGA GTTCC AAGCA GAGGG AGCAC
  CTAGT CATTC TCAGA CCGTT TCTAT CGTCT CGTCT CAAGG TTCGT CTCCC TCGTG


     1380          1390          1400         1410         1420         1430
    *           *            *           *    *           *            *
  AGATG TGAAG GCTGG TGGCA GAGAG CATGG CGCAT CGGGT CGCTG AGGGA TGGAC
  TCTAC ACTTC CGACC ACCGT CTCTC GTACC GCGTA GCCCA GCGAC TCCCT ACCTG


          1440          1450          1460         1470         1480
    *           *            *           *    *           *            *
  AGAGC ATGGA CAGGG AGCAA GGCCA GGCAG GGACA GGGCC AGGTG CGCCC ATGGA
  TCTCG TACCT GTCCC TCGTT CCGGT CCGTC CCTGT CCCGG TCCAC GCGGG TACCT


             >Sau3A1
                |
             >BamH1
                |
     1490          1500          1510         1520         1530         1540
    *           *            |*            *    *           *            *
  AGGAC CTAGG TCTGG ATCCT AAATG CACGG AGAAG TCACT GGAGG GCTTT GGGGC
  TCCTG GATCC AGACC TAGGA TTTAC GTGCC TCTTC AGTGA CCTCC CGAAA CCCCG


          1550          1560          1570         1580         1590
    *           *            *           *    *           *            *
  CAGGC AGTGG TATCA CCGGT CAGCA GTCAT AGAGG GGTGG CCTAG GGGGT GCTGC
  GTCCG TCACC ATAGT GGCCA GTCGT CAGTA TCTCC CCACC GGATC CCCCA CGACG
```

21   Polymorphic site >Apa1   4/5
                                                    |
  1600   1610   1620   1630   1640 | 1650
   *   *   *   *   *   *   *   *   *   * | *
CGTTG AGTGT CTGTG TGGGT GGGGG GTGGT GGGAT TGAGC AGTGA GGGGC CCAGC
GCAAC TCACA GACAC ACCCA CCCCC CACCA CCCTA ACTCG TCACT CCCCG GGTCG

        >Sac1   >Sau3A1
          | |
          1660   1670   1680   1690   1700
   * | *   *   *   *   *   * | *   *   *
TGAGA GCTCC TGTGC CTTCT CTATC CCCGT GCCCA CAGAT CGTCC TGGGG TGCAG
ACTCT CGAGG ACACG GAAGA GATAG GGGCA CGGGT GTCTA GCAGG ACCCC ACGTC
                                        Intron 8 | Exon 9

        Absence of Taq site

          GAT C GAGGCC = Taq1+ allele
                                                      >Pst1
  1710   1720   1730   1740   1750 | 1760
   *   *   *   *   *   *   *   *   * | *   *
GACGC CGCGC TGATT GAGGC CATCC AGGAC CGCCT GTCCA ACACA CTGCA GACGT
CTGCG GCGCG ACTAA CTCCG GTAGG TCCTG GCGGA CAGGT TGTGT GACGT CTGCA
                   Primer 466   primer 455 and

                                                          >Sau3A1
                                                            |
          1770   1780   1790   1800   1810 |
   *   *   *   *   *   *   *   *   *   * | *
ACATC CGCTG CCGCC ACCCG CCCCC GGGCA GCCAC CTGCT CTATG CCAAG ATGAT
TGTAG GCGAC GGCGG TGGGC GGGGG CCCGT CGGTG GACGA GATAC GGTTC TACTA
770 (reverse 455)

  1820   1830   1840   1850   1860   1870
   *   *   *   *   *   *   *   *   *   *   *
CCAGA AGCTA GCCGA CCTGC GCAGC CTCAA TGAGG AGCAC TCCAA GCAGT ACCGC
GGTCT TCGAT CGGCT GGACG CGTCG GAGTT ACTCC TCGTG AGGTT CGTCA TGGCG
primer 467

        1880   1890   1900   1910   1920
   *   *   *   *   *   *   *   *   *   *   *
TGCCT CTCCT TCCAG CCTGA GTGCA GCATG AAGCT AACGC CCCTT GTGCT CGAAG
ACGGA GAGGA AGGTC GGACT CACGT CGTAC TTCGA TTGCG GGGAA CACGA GCTTC
                                        primer 468

        >Sau3A1
          |
  1930   1940   1950   1960   1970   1980
   *   * | *   *   *   *   *   *   *   *   *
TGTTT GGCAA TGAGA TCTCC TGACT AGGAC AGCCT GTGCG GTGCC TGGGT GGGGC
ACAAA CCGTT ACTCT AGAGG ACTGA TCCTG TCGGA CACGC CACGG ACCCA CCCCG

          1990   2000   2010   2020   2030
   *   *   *   *   *   *   *   *   *   *   *
TGCTC CTCCA GGGCC ACGTG CCAGG CCCGG GGCTG GCGGC TACTC AGCAG CCCTC
ACGAG GAGGT CCCGG TGCAC GGTCC GGGCC CCGAC CGCCG ATGAG TCGTC GGGAG
                                                 primer 459

  2040   2050   2060   2070   2080   2090

```
      *        *        *        *        *        *        *        *        *        *        *
    CTCAC   CCGTC   TGGGG   TTCAG   CCCCT   CCTCT   GCCAC   CTCCC   CTATC   CACCC   AGCCC
    GAGTG   GGCAG   ACCCC   AAGTC   GGGGA   GGAGA   CGGTG   GAGGG   GATAG   GTGGG   TCGGG


              2100             2110             2120             2130             2140
      *        *        *        *        *        *        *        *        *        *        *
    ATTCT   CTCTC   CTGTC   CAACC   TAACC   CCTTT   CCTGC   GGGCT   TTTCC   CCGGT   CCCTT
    TAAGA   GAGAG   GACAG   GTTGG   ATTGG   GGAAA   GGACG   CCCGA   AAAGG   GGCCA   GGGAA


      2150..          2160
       .*      *        *        *
    GAGAC   CTCAG   CCATG   AGGAG   TTGC
    CTCTG   GAGTC   GGTAC   TCCTC   AACG
          primer 465
```

Primer underlined on top strand is a forward primer, those on the bottom strand are reverse primers.
Any pair wise combination of these primers or primers based on this and surrounding sequence can amplify the region by polymerase chain reaction.

primer in intron 8(5'-CAGAGCATGGACAGGGAGCAAG-3') and
the other in exon 9 (5'-GCAACTCCTCATGGCTGAGGTCTCA-3'
producing a 740 base pair fragment (Fig. 2).

5   PCR was carried out in a volume of 20 ul
containing 200ng genomic DNA, 20pmol of each primer,
200 uM dNTPs, 50mM KCl, 10mM Tris (pH8.3), 1.5mM, MgCl2
and 1U Taq DNA polymerase (TOYOBO, Osaka, Japan). Each
sample was subjected to 37 amplification cycles as
10   follows: Step 1 - 3 min at 94°C, 1 min at 62°C, 2 min
at 72°C; Step 2 to 6 - 20 sec at 94°C, 20 sec at 62°C,
1 min at 72°C, Step 7 to 36 - 5 sec, 5 sec, 30 sec
respectively. Amplification regimes should be
optimised for any particular thermal cycling device. A
15   10 ul aliquot of each PCR product was digested with 5
units of endonuclease Bsml at 65°C (New England
Biolabs, MA USA), Apal at 37°C or Taql* (Promega Co.
Australia) at 65°C for 1 hour. A clone of an unrelated
gene was used as an internal control for both Bsml and
20   Apal digestion. For Taql digestion, an invariant Taql
site in the PCR product itself was used as an internal
control. The digested PCR products were separated on
1.2% (Bsml and Apal), or 2.0% (Taql) agarose gels
containing 0.5ug/ml ethidium bromide, 0.09M Tris-Borate
25   and 0.002M EDTA, pH8.3 for 1 hr at 100V. EcoRI
digested SPP1 marker (Bresatec Limited, Adelaide,
Australia) was used as the size standard for all
agarose gels. Due to the sequence of the relevant
sites several other restriction enzymes can be used to
30   detect these polymorphisms. Bsml site sequence from an
invariant adjacent Stu-1 site; B allele
AGGCCTGCGCATTCCC, b allele underlined G is an A. This
sequence change can be detected with Aosl, Fspl, Msti,
Fdi2, Hinpl, Hhal and their isoschizomers. Sequence at
35   the polymorphic Apal site ending in an adjacent
invariant Pvu2 site is: A allele GAGGGGCCCAGCTG, in

the a allele the underlined G is a T. The presence of
the G can be detected by Ban2, Aoc2, Pss1, Pa11, Hae3,
Cfr3I, Asu1, Sau96I, Eco0109I, Dra2, and isoschizomers.
The presence of the T creates a polymorphisms for Ban1,
and its isoschizomers. The sequence of the Taq1
polymorphism spanning invariant Hba1 to Hae3 sites is:
T allele GCGCTGAT$\underline{T}$GAGGCC, in the t allele the
underlined T is a C. This polymorphism can be also
detected by Mbo1, Sau3A, Dpn1 and their isoschizomers.

Taq1* RFLP: We have previously reported that Bsm1
and Apa1 RFLPs in the vitamin D receptor gene predict
serum osteocalcin levels. These polymorphic sites are
located in the region of genomic DNA from exon 7 to the
3′ untranslated region (3′-UTR). To characterize the
differences between two common vitamin D receptor gene
alleles (AB and ab), we have sequenced this region in
homozygotes of genotypes AABB, aabb. We have
identified a number of sequence differences, including
15 non coding changes. There is a single synonymous
coding region change, a T for C in an isoleucine codon
(ATT to ATC, isoleucine codons) in exon 9.

Statistical Analysis

Analysis of the variance (ANOVA) was performed
using Statview+Graphics statistical package (Abacus
Concepts, Berkeley, CA, USA) on a Macintosh SE/30
computer. Fisher's protected
least-significant-difference (PLSD) test was used to
assess the relationship between RFLP and the BMD,
height, weight. Significance levels quoted are for the
initial F tests on the null hypothesis (no difference
between the means) of the overall effect and for the
confidence level of the pairwise comparison of the
continuous variable means of each categorical (RFLP)
class. Students t-test was used for pairwise
comparisons. Relationships of continuous and
categorical variables were established by multiple

regression. Relationships between RFLP markers were established by contingency tables and Chi square.

Results

The frequencies of these three RFLPs in 535 subjects are shown Table 6. The RFLPs were coded as Bb (Bsml), Aa (Apal) and Tt (Taql), where the uppercase letter signifies absence of the site and lowercase signifies presence of the site. The frequencies of Bsml and Apal RFLP are similar to that set out above (Table 1). RFLPs had a high degree of coassociation (Table 7). The AA genotype is highly associated with BB and tt at frequencies 92.7% and 95.3%, respectively; correspondingly, aa was found with bb and TT at frequencies of 61.6% and 65.3%, respectively. Comparing Bsml with Taql RFLP, tt, Tt, and TT genotypes are highly associated with BB, Bb and bb at frequencies 95.5%, 95.1% and 96.4% respectively. Because the Bsml and Taql results are so closely correlated, in subsequent discussions we have equated Bsml and Taql results and will refer only to Bsml results.

The relationship between RFLPs and BMD at both LS and FN sites were analyzed in the 535 subjects. The distribution of this population with respect to age, height, weight, and menopausal status is shown in Table 8. Age, height, and weight were not significantly related to any RFLP genotype (Table 9). In females, mean LS BMD of the BB and AA group are 9.9% (1.017 vs 1.118) and 8.6% (1.049 vs 1.139) lower than those of the bb and aa groups respectively. The FN BMD of the BB and AA groups are also 5.6% and 5.3% lower than those of the bb and aa groups respectively. A heterozygote effect indicating co-dominance of alleles was also observed (Figure 3). Lower LS and FN BMD were associated with the absence of both restriction site alleles (BA). The differences of mean BMD at LS and FN between BBAA genotype and bbaa genotype was wider

Table 6

Frequencies of RFLPs in study population.

Genotype	N	Frequency %	Allele
BB	89	16.8	B=0.418
Bb	266	50.1	b=0.582
bb	176	33.1	
AA	133	25.5	A=0.512
Aa	268	51.3	a=0.488
aa	121	23.2	
TT	188	35.4	T=0.596
Tt	257	48.4	t=0.404
tt	86	16.2	

Table 7

RFLP markers have a high degree of coassociation. Bsm-1 genotypes tabulated with Apa-1 and Taq1 genotypes. n refers to number of individuals. Chi square value and p value reflect the rejection of the null hypothesis of no association between the markers.

Marker	BB	Bb	bb	total n
AA	78	44	11	133
Aa	5	206	56	267
aa	4	8	106	118
total n	87	258	173	518

Chi^2=428   p=0.0001

Marker	BB	Bb	bb	total n
TT	1	14	173	188
Tt	5	249	3	257
a	83	3	0	86
total n	89	266	176	531

Chi^2=912   p=0.0001

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Table 8

Population characteristics of study group

Sex	Number
Males	88
Females	447
Premenopausal	185
Postmenopausal	262
Mean years since menopause $\pm$ SEM	11.3 ± 0.07

Mean values of anthropomorphic parameters in total subjects ($\pm$SEM).

Age (year)	49.6+0.6
Height (cm)	163.8+0.4
Weight (kg)	64.8+0.5

Table 9

Mean values of anthropomorphic parameters according to Bsm-1 genotype

Genotype	n	Age	Height	Weight
Females				
BB	75	50+2	162+1	62+1
Bb	216	51+1	161+1	63+1
bb	154	52+1	161+1	64+1
p value		0.6	0.3	0.9
Males				
BB	14	35+4	177+2	72+10
Bb	50	41+2	176+1	75+10
bb	22	42+4	176+2	74+14
p value		0.9	0.6	0.4

Notes: n refers to number, p is the value for the overall effect of genotype on the variable in question, derived from ANOVA. All p values indicate no significant differences in mean values between different genotypes.

(13.4%, 7.8% respectively) than those of BB and bb or AA and aa (Table 9).

The effect of genotype was assessed by multiple regression analysis of covariance including age(yr), menopausal status (year post menopause; YPM), height (cm), weight (kg) and Bsm1 genotype (BB=1, Bb=2, bb=3) in females, giving the equation; LS BMD (g/cm2) = 0.419+0.054 Bsm1 genotype -0.004age -0.994 YPM+0.02 weight + 0.004 height (n=425) r=0.58 R2=0.34,

	Bsm1	age	YPM	Weight	Height
p value	0.0001	0.0013	0.0001	0.0045	0.003
F-score	24.9	16.0	10.4	8.2	8.9

FN BMD g/cm2)=0.456+0.025 Bsm1 genotype -0.004 age -0.004YPM + 0.04 weight + 0.02 height (n=425) 4=0.68, R2=0.47

	Bsm1	age	YPM	Weight	Height
p value	0.002	0.0001	0.0004	0.0001	0.022
F-score	9.6	41.7	12.8	35.2	5.3

Both lumbar spine and femoral neck BMD were negatively and independently correlated with the menopausal status, age, Bsm1 RFP was also correlated independently with BMD at LS and F in females. Male's results were as follows:

LS BMD (g/cm2)=1.039+0.058 Bsm1 genotype (n=85)
r=0.22 RS=0.05, p=0.038, F-score 4.9

FN BMD(g/cm2)=1.046 -0.003age (n=85)
r=0./32, R2=0.10, p=0.017, F-score 5.9

Intercept with the Fracture Threshold

A value of lumbar spine BMD, below which a heightened risk of osteoporotic fracture exists, was derived form a large cross sectional study in the city of Dubbo Australia. This value 0.97 gm.cm2 is similar to a fracture threshold described from an American population. Clearly, if VDR genotype affects BMD and

subsequently osteoporosis susceptibility, a difference
in the intercept of the age related change in bone mass
and the fracture threshold should be apparent between
genotypes. Figure 5 shows simple age related

5   regression lines for female LS BMD of BB, Bb and bb
genotypes intersecting the fracture threshold value. A
comparison between BB and bb reveals a 10 year
difference in the intercept (60.3 yr versus 71.1 year,
respectively) with an intermediate value for the Bb

10   heterozygotes (68.1 year). A similar result was
apparent for the neck of femur (Figure 6) using a
fracture threshold of 0.7 gm/cm^2 (BB, 66 years; Bb, 70
years; bb, 74 years).

STUDY 3

15   The effect of the common VDR gene alleles on bone
density was examined using the twin model, in which
within-pair comparisons eliminate age and various
cohort confounders. 250 Caucasian twins were studied
comprising 70 MZ and 55 DZ twin pairs, including 7 male

20   MZ pairs and 6 male DZ pairs, aged between 17 and 70
years; MZ 45$\pm$13 yrs and DZ 44$\pm$11 yrs, mean $\pm$ SD. Bone
density was measured at the lumbar spine and proximal
femur with a Lunar DP3 dual-photon absorptiometer
(LUNAR Corporation, Madison, WI) or Lunar DEXA dual

25   energy X-ray absorptiometry as previously described
(Pocock et al 1987). All female twin pairs were
concordant for menopausal status and if post
menopausal, for years since menopause.
        The VDR gene in the region bearing the polymorphic

30   sites for the Bsm-1, Apa-1 and EcoRV sites previously
shown to predict differences in bone turnover markers
was sequenced. These sites are in the region of the
gene from exon 7 to the 3'-UTR. None of the
polymorphic sites was in the coding region or involved

35   potential splice sites and the highly informative Bsm-1
site was found to arise from a G for A substitution in

intron 8. There was only one difference in the coding
region between the two most common allelic forms. This
included a T for C substitution in exon 9, changing ATT
to ATC, without changing the encoded amino acid
sequence (isoleucine). The DNA sequence flanking the
Bsm-1 site was used in a polymerase chain
reaction-based method to amplify a 2.1 - 2.2 kb
fragment from exon 7 to exon 9 to facilitate genotyping
of subjects. PCR amplification of leucocyte DNA was
performed with a Corbett FTS-1 Thermal Sequencer
(Corbett Research, Mortlake NSW, Australia) PCR
instrument using primers
     5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' and
     5'-AACCAGCGGGAAGAGGTCAAGGG-3' prior to
endonuclease digestion with Bsm-1 (New England Biolabs
Inc, Gene Search, Brisbane, Australia). The presence
of the Bsm-1 site cuts a 825 bp product to 650 bp and
175 bp fragments. A 4.7 kb plasmid with a single Bsm-1
site, which linearises with Bsm-1 digestion, was used
as an internal control to avoid misassignment of the
allelic forms due to partial digests.
     From twin studies the within pair difference in
BMD (ΔBMD%) at the lumbar spine and proximal femur was
examined in relation to allelic variation in DZ twin
pairs (Figure 7). In both regions this was
significantly less in DZ twins concordant compared to
those discordant for VDR gene alleles. The ΔBMD% for
lumbar spine in the MZ twins was not significantly
different from that in the DZ twins concordant for the
VDR alleles, both of which were statistically different
from those in DZ twins discordant for the alleles (p<
0.0001). Similar but weaker effects in the proximal
femur are consistent with stronger environmental
influences on bone density in this region. Limiting
the analysis to premenopausal twins did not alter the
results. Controlling for potential confounding by

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anthropomorphic features of height and weight, VDR
genotype remained the strongest predictor at the lumbar
spine (p=0.0002) and the trochanteric region (p=0.02)
although not at the neck region of the proximal femur.

5   In view of the previously demonstrated co-dominant
effect of Bsm-1 alleles on bone turnover indices, we
would expect a co-dominant effect on the bone mass
trait with a linear relationship between the degree of
difference in genotype and the difference in trait

10   within twin pairs (Figure 8). According to the
sib-pair linkage analysis approach, a significant
correlation between the squared difference in a trait
and the proportion of identical genes within a sibling
pair indicates genetic linkage. By this analysis the

15   VDR gene alleles were co-dominant at the lumbar spine
and most sites in the proximal femur. Comparing the Δ
BMD% with respect to degree of concordance for VDR
alleles showed 1.5 to 2.5-fold greater within pair
differences for the discordant twins (see Figures 7 and

20   8). In 21 of 22 dizygotic twin pairs discordant for
the VDR alleles, the b allele was associated with
higher bone density (Figure 9A). In premenopausal
females (randomly selected as singletons from MZ and DZ
twin pairs), the VDR bb genotype was also associated

25   with higher bone density (Figure 9B) while the BB
genotype was associated with lower bone mass with a
clear codominant effect between the alleles (Figure
9B).

     These data demonstrate that the differences of VDR
30   gene alleles indicate a major proportion of the
differences in bone density in a population of normal
individuals. The BB,AA,EE and/or tt VDR genotypes are
associated with low BMD in both females and males. VDR
gene RFLPs genotypes are therefore useful predictors of

35   propensity to high bone turnover and low bone mass,
physiological variability not only in peak bone mass

but also bone mass in later life in both females and males. Until now, the mechanisms of the genetic effects on bone density and bone turnover have been unclear. However, 1,25-dihydroxyvitamin D is a
5   enhancer of osteocalcin synthesis through the vitamin D responsive element in the promotor of the VDR gene (Morrison 1989 Science). The present inventors have also shown that common allelic variants of the VDR gene are associated with differences in the serum
10   osteocalcin levels. Moreover, these allelic variants of the VDR gene predict the difference in bone density between dizygotic twin pairs.

It is concluded that these VDR gene RFLP's are markers for physiological variability in bone mass in both females and males. The present inventors have found that Bsm1 RFLP correlated independently with BMD at LS and FN.

Table 10. Age and years since menopause (YSM) amongst twins. A, those DZ twins concordant and discordant for VDR gene alleles; B; individuals with differing alleles for the VDR gene. All values are expressed as means ± SD.

A

	Concordant	Discordant
Age	41.1 ± 10.6 n = 30	45.5 ± 12.3 n = 23
YSM	4.4 ± 3.6 n = 5	9.3 + 5.3 n = 7

B

	BB	Bb	bb
Age	44.0 ± 12.8	43.6 ± 13.4	45.7 ± 11.3
YSM	9.0 ± 2.5 n = 9	11.8 ± 9.5 n = 12	9.8 ± 7.8 n = 7

Table 11A. Correlation co-efficients between monozygotic and dizygotic twin pairs with dizygotic pairs further segregated into those concordant and discordant for vitamin D receptor genotype.

Variable	rMZ	rDZ	p	rDZ concordant	rDZ discordant	p
Lumbar Spine	0.81	0.16	<0.0001	0.41	0.04	<0.001
Femoral Neck	0.79	0.43	<0.0008	0.44	0.41	NS
Ward's Triangle	0.83	0.51	<0.004	0.43	0.54	NS
Trochanteric	0.82	0.34	<0.0002	0.49	0.38	<0.006
Weight	0.80	0.42	<0.0004	0.36	0.45	NS

Notes: p; denotes the p value for the test that correlation coefficients are significantly different. In the twin model a significant difference between rMZ and rDZ is evidence for a genetic effect on the trait in question. In our comparison of DZ twin pairs with the same within-pair VDR genotype (DZ-concordant) or different within-pair VDR genotype (DZ-discordant), a significantly different correlation between rDZ-concordant and rDZ-discordant is supportive of a contribution of the VDR genotype to the genetic effect on the trait in question.

Table 11B. Within-twin pair proportional difference in bone mineral density according to zygosity and degree of discordance of the twin pair of alleles of the vitamin D receptor gene. Values of percentage differences are means $\pm$ sem.

Variable	n	Lumbar spine	Femoral Neck	Ward's Triangle	Trochanteric region
MZ	69	6.0 ± 0.7	8.1 ± 0.9	10.0 ± 1.2	9.3 ± 1.0
DZ-all	55	11.9 ± 1.4	12.1 ± 1.4	15.9 ± 2.0	13.5 ± 1.7
DZ-concordant	33	7.5 ± 1.3	10.2 ± 1.5	12.4 ± 2.0	9.7 ± 1.7
DZ-discordant	22	18.5 ± 2.5	15.1 ± 2.8	20.6 ± 3.8	18.7 ± 3.0
Genetic effect		75%	48%	59%	90%
DZ-discordant:					
-1 allele	14	15.6 ± 2.5	13.2 ± 2.7	16.4 ± 3.7	19.9 ± 2.8
-2 alleles	8	22.0 ± 5.2	17.9 ± 5.9	28.5 ± 8.2	16.4 ± 7.0
DZ-discordant/ DZ-con		2.47	1.48	1.66	1.93
DZ-2 alleles/ DZ-con		2.93	1.75	2.30	1.71

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Importantly, the homozygous BBAA or AAtt genotype
are associated with low bone density, and mean BMD at
the LS and FN site were about 12% and 8% lower in BBAA
homozygotes compared with bbaa genotype in both females
5   and males. These genotypic differences are important
for later life, because these differences of BMD
indicate a 10 year difference in the fracture threshold.
These allelic differences provide a mechanism for the
genetic effect on bone mass observed in twin studies and
10   provide a simple genetic test of carrier status for low
bone mass alleles. Identification of the vitamin D
receptor genotype as an important determinant of bone
mass may open new avenues for prevention and therapy for
osteoporosis.

15   Demonstration of Differences in Response to Treatment
in Different Gene Types.

Data described above have demonstrated that the
VDR alleles described above are functionally different.
It would therefore be expected that individuals of
20   different genotype would exhibit different responses to
treatment with calcitriol and/or analogues. This was
confirmed by examining responses to calcitriol
administration in 10 normal young females of each
homozygous Bsm 1 genotype (BB and bb) and analysing
25   responses to treatment in three markers of bone calcium
metabolism; osteocalcin, parathyroid hormone and
urinary calcium (see Fig. 10).

Osteocalcin serum levels were different (p<0.01)
at basline in the BB and bb groups. The BB genotype
30   again had the higher osteocalcin. After calcitriol
treatmen: the bb group had a higher percent response
from baseline than the BB group. Although the BB group
had a lesser percent response, since they had a higher
baseline osteocalcin, the total response was higher.

35   Parathyroid hormone is known to be repressed by
calcitriol, however, the extent of repression by

calcitriol treatment was significantly different in the
two genotypic groups. Parathyroid hormone was weakly
repressed in the BB group and strongly repressed in the
bb group, indicating substantial differences in the
response of PTH to calcitriol therapy. Total urinary
calcium excretion over the treatment period (area under
the curve) was significantly higher in the BB group than
the bb group indicating different calcium handling
responses according to genotype. the reduced repression
of parathyroid hormone in the face of calcitriol
treatment, coupled with increased urinary calcium output
indicates different calcium homeostatic mechanisms,
compatible with mobilisation of skeletal calcium.

VDR and Other Conditions

The vitamin D receptor and vitamin D endocrine
system are implicatedin several other pathological and
physiological states. Such differences in the vitamin D
receptor gene, leading to different responses to
endogenous calcitriol, exogenous calcitriol and therapy
using vitamin D analogues, will also result in
differences in progression and susceptibility to other
disorders where a significant component of regulation is
effected by calcitriol. Known examples of conditions
and diseases where the vitamin D endocrine system and
VDR mediated events occur include AIDS virus (HIV-1)
replication, breast cancer cell proliferation, colonic
cancer cell growth, keratinocyte differentiation,
psoriasis cell replication and function,
spermatogenesis, melanoma and other tumours.

As a result of the invention described herein, it
is therefore obvious that functionally different alleles
of the VDR could affect the susceptibility, progress,
prognosis and therapeutic efficacy of various
treatments, in such diseases and conditions where the
vitamin D receptor and vitamin D endocrine system are
known to regulate aspects of the disease process. While

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these are examples of physiological and disease
processes influenced by the vitamin D endocrine system,
it is in no way exclusive of other processes influenced
by the vitamin D endocrine system. Given the data
5   described herein, it is obvious that all physiological
and disease processes known to be influenced by the
vitamin D endocrine system, as described in a recent
comprehensive review by Walters, M (newly identified
actions of the vitamin D endocrine system; Endocrine
10   reviews, 13:719-764) and papers referred to therein,
could be assessed and investigated in the way described
herein, and that these could be influenced by the
vitamin D receptor genotype and therefore the genotype
of an individual will be of importance to the prognosis,
15   progression, susceptibility and treatment of all
conditions and diseases in which vitamin D receptor and
the vitamin D endocrine system are involved.

     Irrespective of the physiological mechanism, these
data have identified for the first time a gene involved
20   in the regulation of bone density. Importantly the
magnitude of the effect is such that it explains the
majority of the strong genetic effect on bone density
and indeed more than half of the adjusted population
variation in bone density. These findings, which will
25   allow earlier interventions in those at increased risk
of osteoporosis, provide important insight into the
mechanism of the wide population variance in bone
density and open the way to development of novel
specifically targeted therapies. This single gene with
30   pleiotropic transcriptional activities is a model for
many pathophysiological processes previously considered
subject to complex multi-factorial genetic regulation.

     This study describes a functional definition of
naturally occurring alleles of a trans-acting
35   transcriptional activator by correlation with the
product of a target gene. The data also indicate that

the receptor allelic differences also relate to major
differences in a target organ - i.e., bone density.
This method of genetic analysis provides a paradigm for
the investigation of the functional significance of
natural allelic variation within the genes of the
ligand-activated receptor superfamily, which can
contribute substantially to a more complete
understanding of the steroid hormone endocrine system.
It is also applicable to the genes for trans-acting
regulators of all kinds.

Genotypic variations in transcriptional regulators
of genes encoding regulatory and/or structural
proteins, determine physiological set-points and
predisposition to pathophysiological states with
implications for susceptibility to disease and for
determining likely responses to therapy. These
genotypic variants are a general model for use in the
determination of disease risk and for choice of therapy
in prevention and treatment.

As a specific example of this model, allelic
variants in the vitamin D receptor gene determine bone
turnover, bone mass and sensitivity to environmental
factors. As such these variants are markers of risk of
development of osteoporosis and indicate likely response
to various modalities of therapy.

The inventors have identified RFLP markers that
define functionally different vitamin D receptor
alleles. The RFLPs herein described are physical
markers that are linked to genetic phenomena. The
inventors advise that it is now obvious that any other
RFLP, physical marker, polymorphic sequence, or genetic
effect detectable in the vitamin D receptor gene or
flanking DNA, which is in linkage with the currently
defined markers, could provide the same information
content as the markers herein described, dependent on
the extent of linkage between the markers defined herein

and any other such marker, consisting of RFLP, physical,
polymorphic sequence, or genetic effect. The inventors
thereby state that other markers, known or unknown, in
linkage with the markers herein described, represent a
5   claimed usage of this invention.

      It will be appreciated by persons skilled in the
art that numerous variations and/or modifications may be
made to the invention as shown in the specific
embodiments without departing from the spirit or scope
10   of the invention as broadly described. The present
embodiments are, therefore, to be considered in all
respects as illustrative and not restrictive.

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CLAIMS:-

1.   A method of assessing an individual's
predisposition to low or high bone density, development
of high or low bone turnover and/or responsiveness to
therapy comprising analysing allelic variation in
relation to the vitamin D receptor gene of the
individual.

2.   A method as claimed in claim 1 in which the
analysis comprises restriction fragment length
polymorphism using endonuclease digestion.

3.   A method as claimed in claim 2 in which a segment
of the vitamin D receptor is amplified using polymerase
chain reaction prior to endonuclease digestion.

4.   A method as claimed in claim 2 or claim 3 in which
the endonuclease is selected from the group consisting
of Bsml, Apal, EcoRV, Taql, and isoschizomers thereof.

5.   A method as claimed in claim 4 in which the
restriction endonuclease is Bsml.

6.   A method as claimed in any one of claims 3 to 5 in
which the segment of the vitamin D receptor is
amplified using a pair of primers selected from the
group consisting of

         5' - CAACCAAGACTACAAGTACCGCGTCAGTGA - 3'
         and 5' - AACCAGCGGAAGAGGTCAAGGG - 3';
         and 5' - CAGAGCATGGACAGGGAGCAAG - 3'
         and 5' - GCAACTCCTCATGGCTGAGGTCTCA - 3'.

7.   A method as claimed in any one of claims 1 to 6 in
which the segment of the vitamin D receptor gene
analysed represents a variableportion of the vitamin D
receptor or gene regions in linkage with at least one
of the Bsml, Apal, EcoRV and Taql cut sites.

8.   A primer pair derived from the sequence of the VDR
gene shown in Table 5 for use in amplifying a segment
of the VDR gene using polymerase chain reaction, the
segment including at least one of the Bsml, Apal or

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Taq1 cut sites as shown in Table 5 or a gene segment in
linkage with at least one of these cut sites.

9.   A primer pair as claimed in claim 8 in which the
primer pair is

         5' - CAACCAAGACTACAAGTACCGCGTCAGTGA - 3' and

         5' - AACCAGCGGAAGAGGTCAAGGG - 3', or

         5' - CAGAGCATGGACAGGGAGCAAG - 3' and

         5' - GCAACTCCTCATGGCTGAGGTCTCA - 3'.

10.   A method of assessing an individual's
predisposition to a pathophysiological state and/or
likely response to therapy comprising analysing
genotypic variations in transcriptional regulators of
genes encoding regulatory and/or structural proteins.
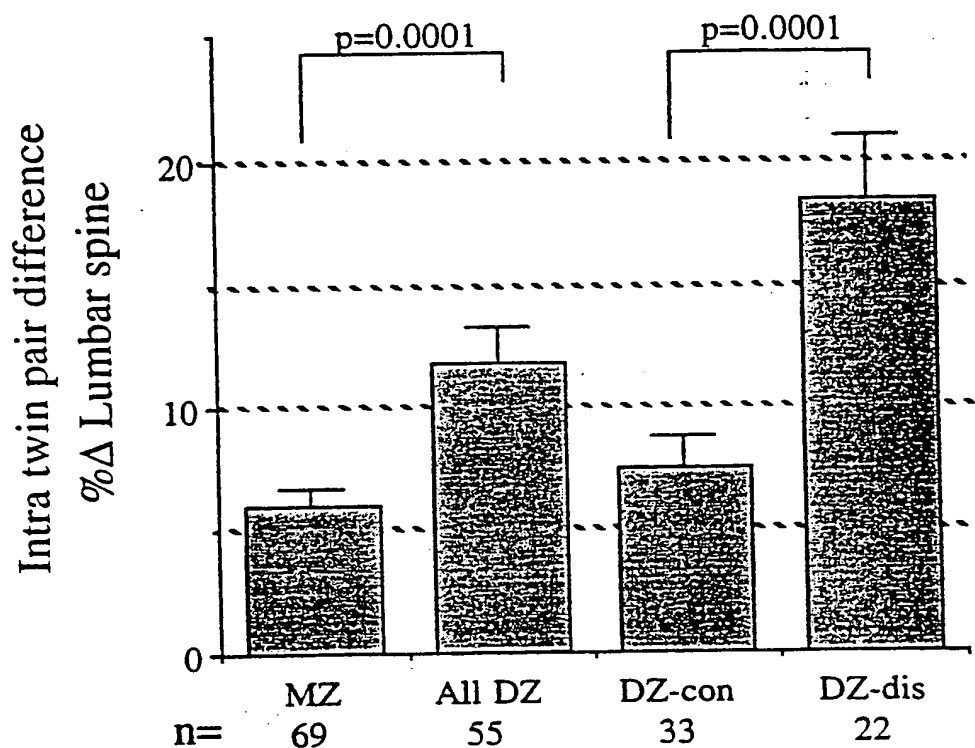
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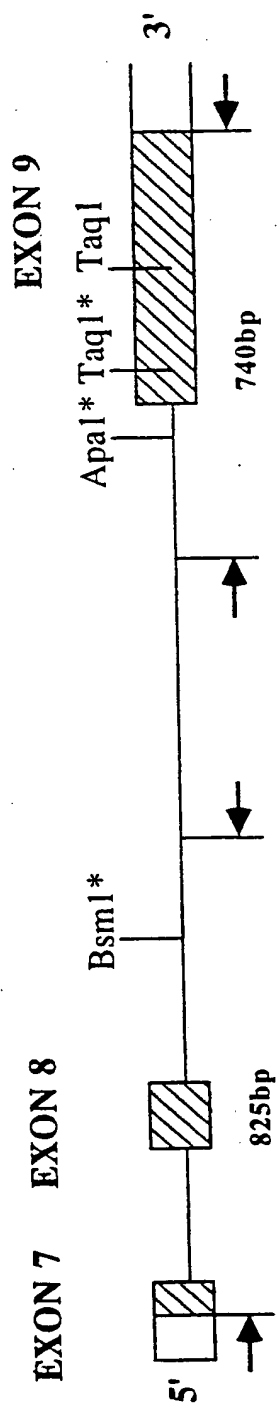
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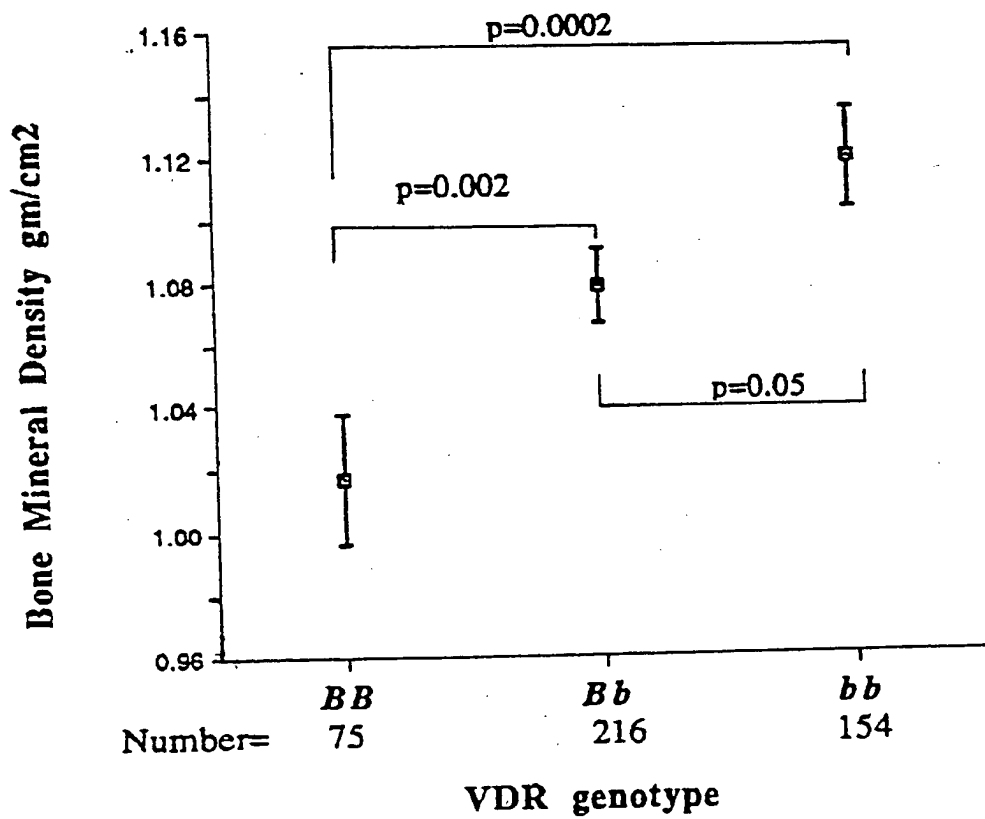
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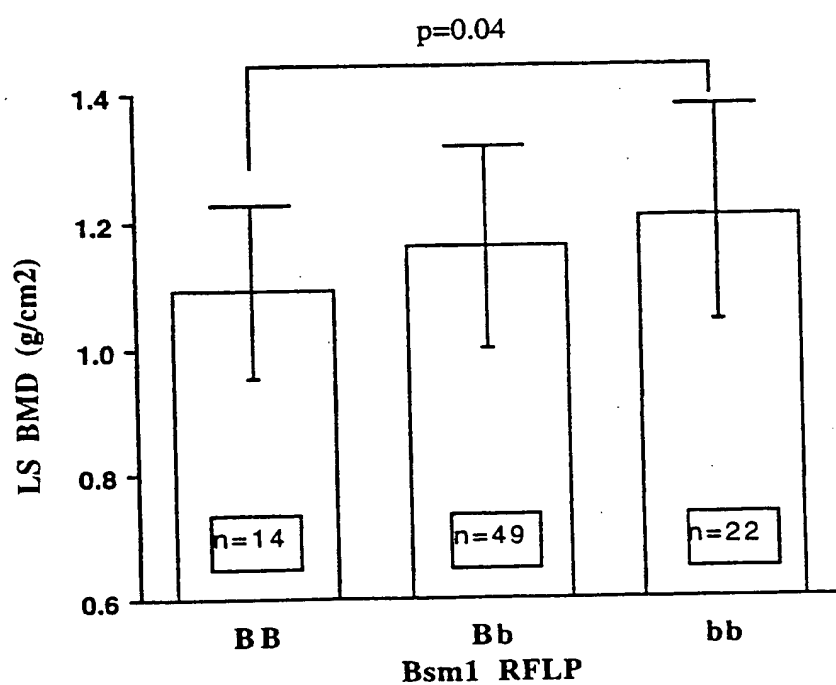
FIG. 4
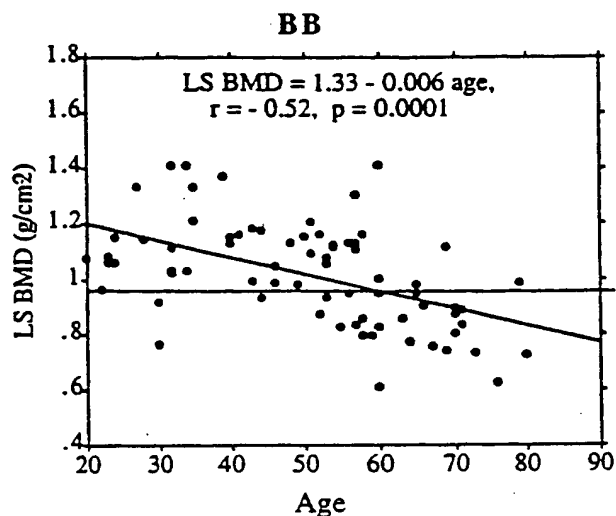
FIG. 5A
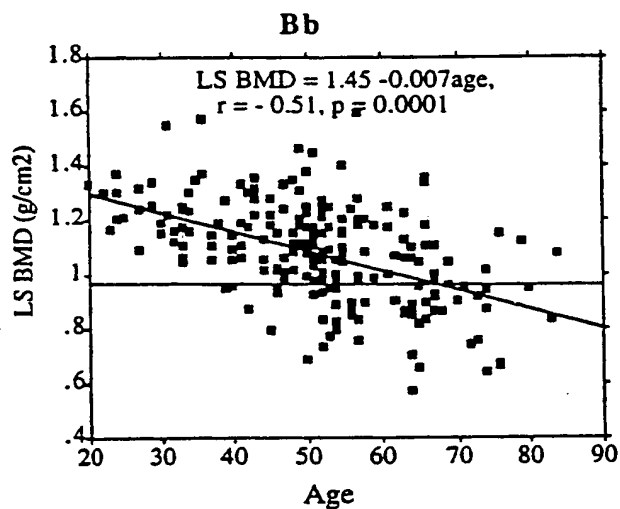
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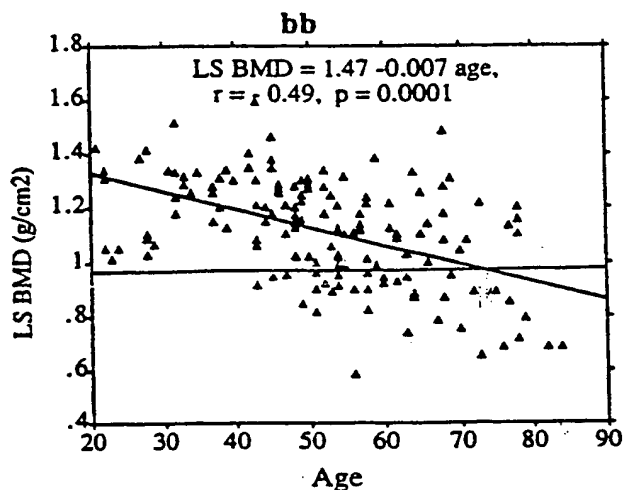
FIG. 5C

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BB

FIG. 6A

Bb
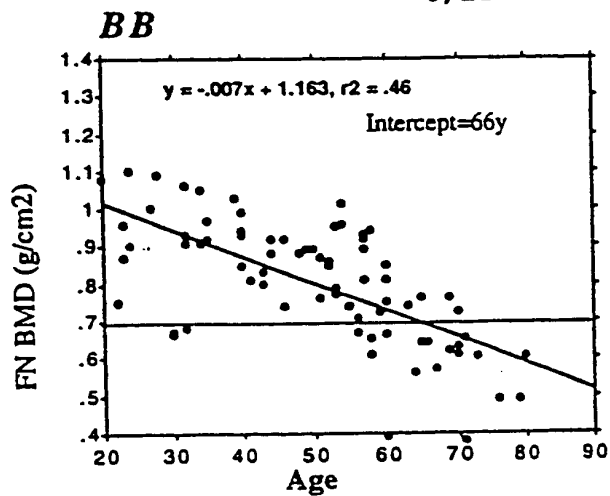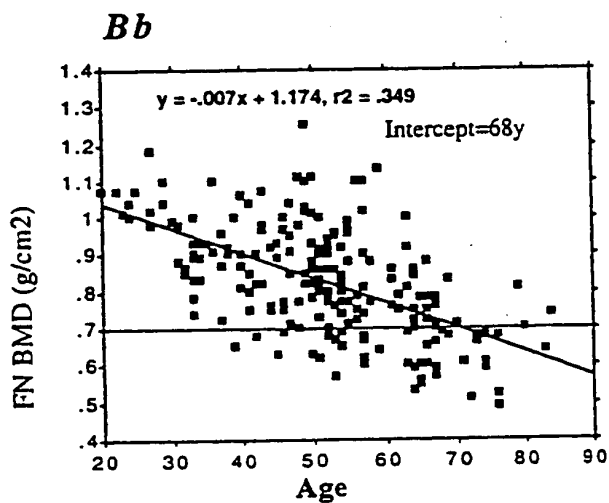
FIG. 6B

bb
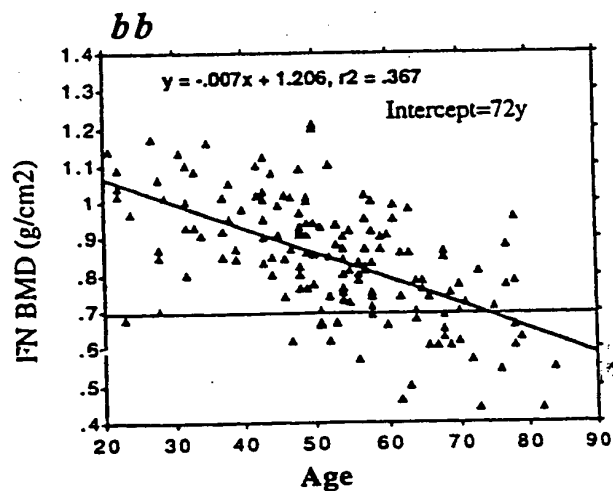
FIG. 6C
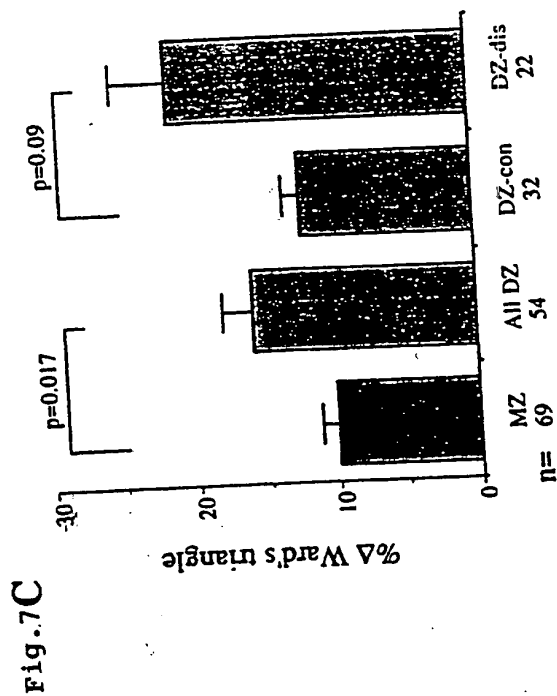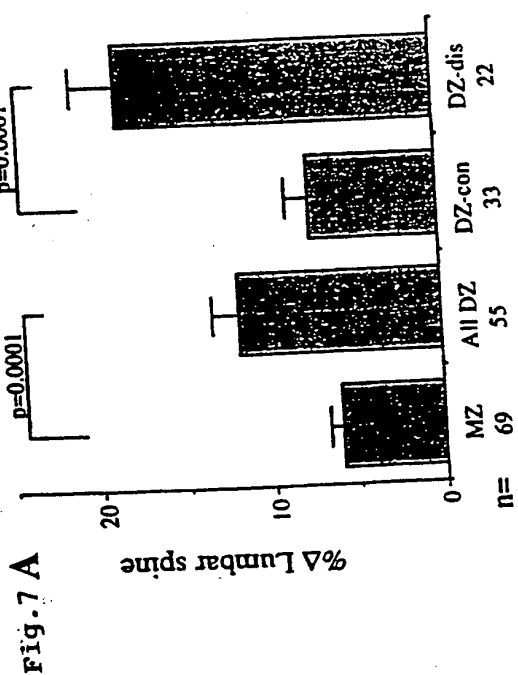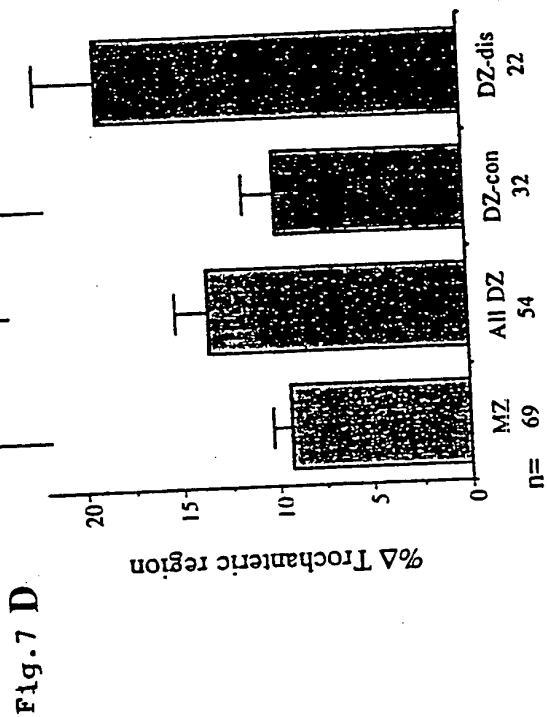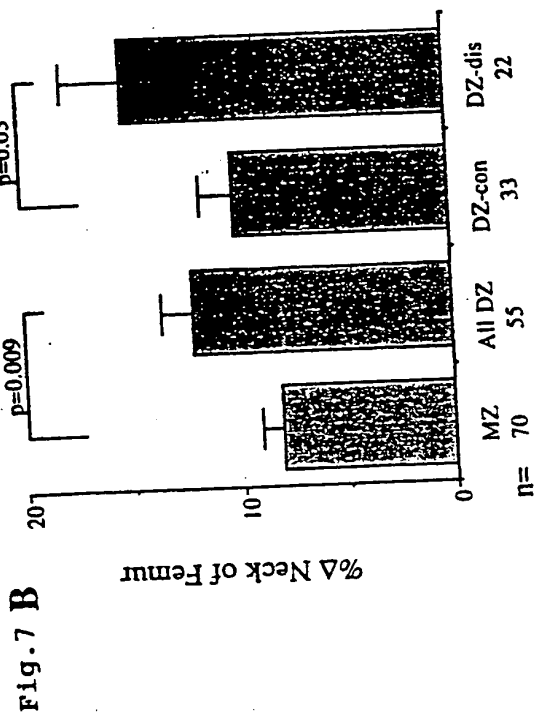
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Fig.7 A

Fig.7 B

Fig.7C

Fig.7 D

Fig. 8 A

Fig. 8 B

Fig. 8 C

Fig. 8 D

Lumbar spine BMD
(gm/cm ^{2})

1.6

1.4

1.2

1.0

0.8

0.6

BB   *Bb*   *bb*

Twin pair genotype.
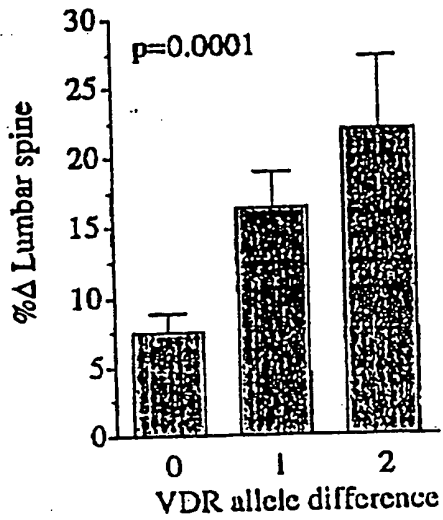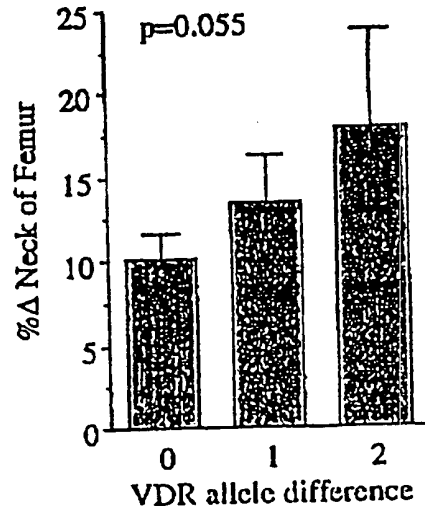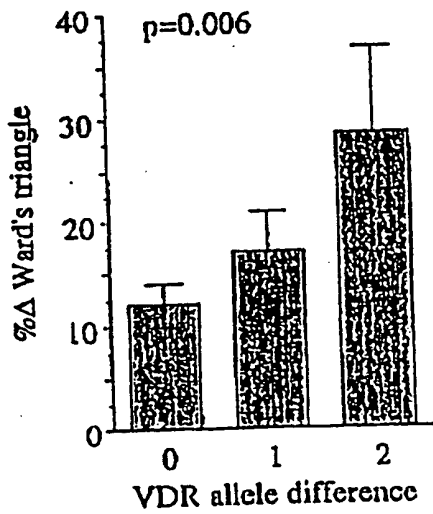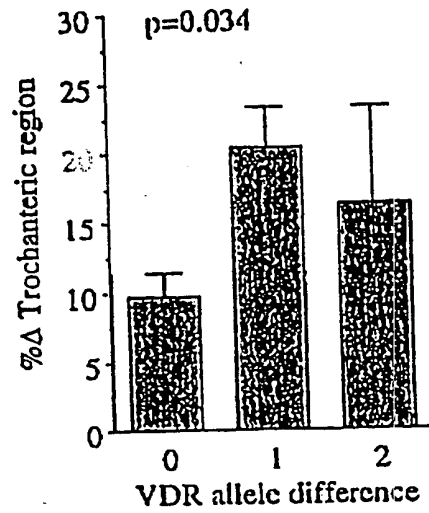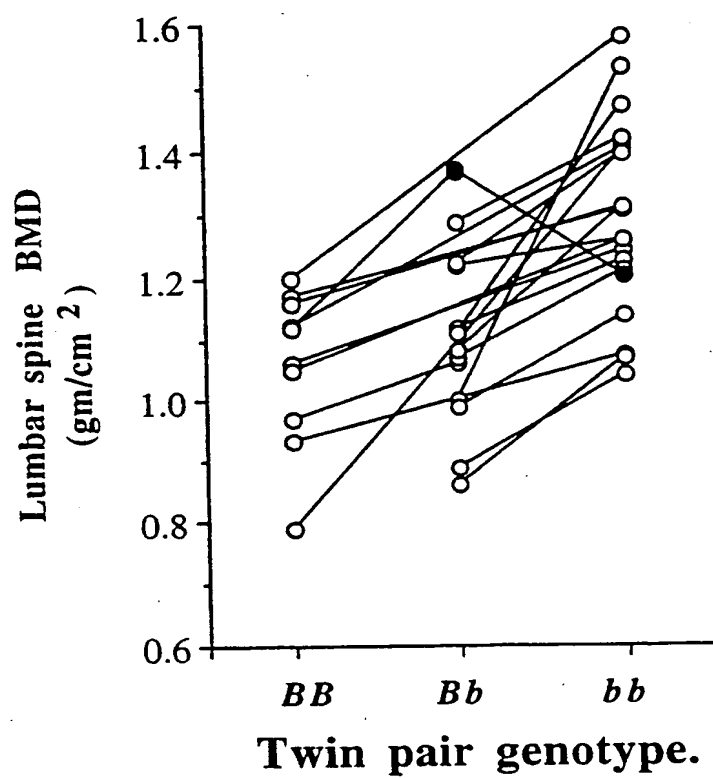
FIG. 9A
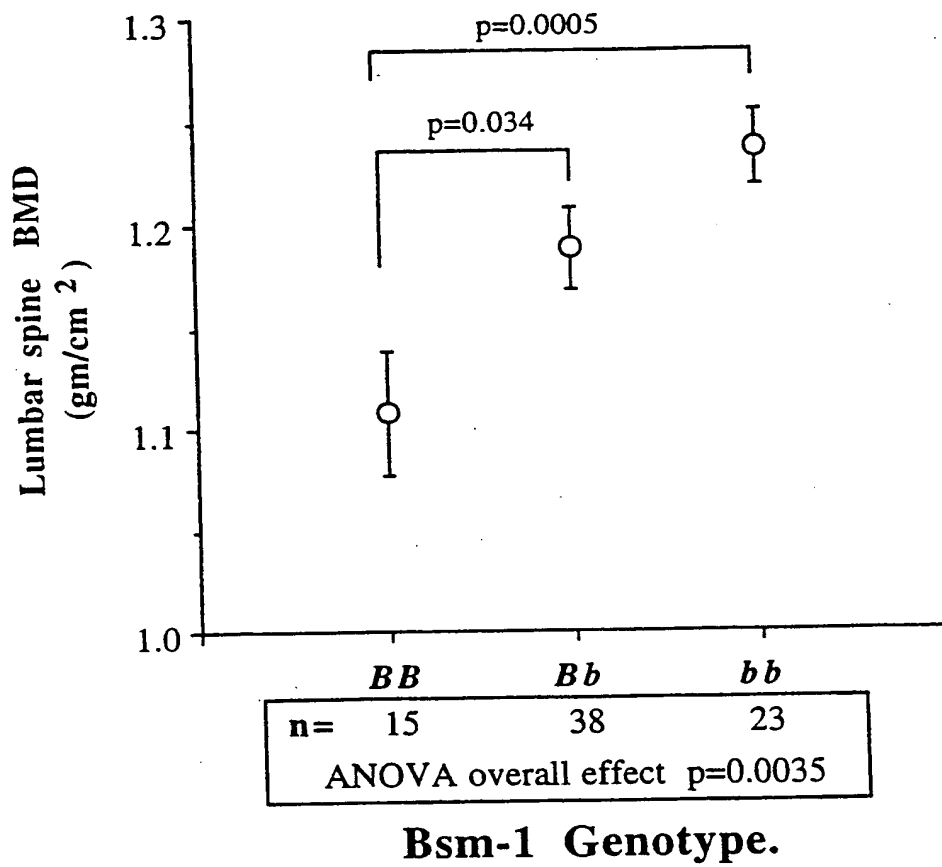
FIG. 9B

FIG. 10A

FIG. 10B

FIG. 10 C

A.   CLASSIFICATION OF SUBJECT MATTER

Int. Cl.^{5} C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC : C12Q, WPAT, CASM, BIOT, STN (See keywords below)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC AU : C12Q 1/68 (020) + C12Q 1/68 (92-93)

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
WPAT : Vitamin ( ) D, vit ( ) D, calciferol, VDR, bone or osteo: G01N 33/1C, C12Q 1/1C; VARIA: +
TRANSCRIPTION: (S) REGULAT:
CHEM ABS: AS ABOVE + 03/CC + 14/CC, BIOT: AS ABOVE, STN: PRIMERS OF CLAIM 6

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Proc. Natl. Acad. Sci. USA. vol. 89, August 1992 Morrison, N.A. et al.; "Contribution of trans-acting factor alleles to normal physiological variability : Vitamin D receptor gene polymorphisms and cirulating osteocalcin," pages 6665-6669.	1, 2, 4, 5, 7, 8, 10
P,X	Proc. Natl. Acad. Sci. USA. vol. 90, March 1993, Bortell R et al; "Constitutive transcription of the osteocalcin gene in osteosarcoma cells is reflected by altered protein - DNA interactions at promoter regulatory elements", pages 2300-2304.	1, 10

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

*	Special categories of cited documents :	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 November 1993 (09.11.93)	18 NOV 1993 (18 . 11 . 93)
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA	CARMELA MONGER
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Form PCT/ISA/210 (continuation of first sheet (2)) (July 1992) cophin

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	Molecular endocrinology, vo. 3(4), 1989, McDonnell D.P. et al; "Functional domains of the human vitamin D_3 receptor regulate osteocalcin gene expression", pages 635-644.	2-9
X Y	Molecular endocrinology, vol. 6(4), 1992, Demay, M.B. et al; "Characterization of 1,25-Dihydroxyvitamin D_3 receptor interactions with target sequences in the rat osteocalcin gene", pages 557-562.	1, 10 2-9
X	AU, 17280/88 (Biotechnology Research Partners Ltd) 30 April 1987 (30.04.87).	10
X	AU,B, 57760/86 (598192) (Biotechnology Research Partners Ltd) 17 April 1985 (17.04.85).	10
X	AU 33223/93 (The Board of Trustees of the Leland Stanford Junior University), 17 December 1991 (17.12.91).	10
X	AU 62127/90 (Imperial Chemical Industries PLC) 7 September 1989 (07.09.89)	10
A	The Journal of Immunology, vo. 148(4) 1992, Shewey L.M. et al; "Differential expression of related HLA class 11 DQß genes caused by nucleotide variation in transcriptional regulatory elements," pages 1265-1273.	
A	Proc. Natl. Acad. Sci. USA. vol. 89, Bortell R. et al., "Vitamin D - responsive protein - DNA interactions at multiple promoter regulatory elements that contribute to the level of rat osteocalcin gene expression", pages 6119-6123.	